The cysteine residue of the SoxY protein as the active site of protein-bound sulfur oxidation of *Paracoccus pantotrophus* GB17

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Abstract Four proteins of *Paracoccus pantotrophus* are required for hydrogen sulfide-, sulfur-, thiosulfate- and sulfite-dependent horse heart cytochrome *c* reduction. The lack of free intermediates suggested a protein-bound sulfur oxidation mechanism. The SoxY protein has a novel motif containing a cysteine residue. Electrospray ionization and matrix-assisted laser desorption ionization mass spectrometry of the SoxYZ protein revealed one mass for SoxZ and different masses for SoxY, indicating native SoxY (10 977 Da) and SoxY with additional masses of +32, +80, +112 and +144 Da, suggesting addition of sulfur, sulfite, thiosulfate and thioperoxomonosulfate. Reduction of SoxY removed the additional masses, indicating a thioether or thioester bond. *N*-Ethylmaleimide inhibited thiosulfate-oxidation and the kinetics suggested a turn-over-dependent mode of action. These data were evidence that the sulfur atom to be oxidized was covalently linked to the thiol moiety of the cysteine residue of SoxY and the active site of sulfur oxidation.

Key words: SoxYZ protein; *S*-Cysteinesulfate; *S*-Thiocysteine; *S*-Thiocysteinesulfate; Sulfur oxidation; *Paracoccus pantotrophus*

1. Introduction

The biological oxidation of hydrogen sulfide to sulfuric acid is a major reaction of the global sulfur cycle and is mediated by litho-, photo- and methylotrophic bacteria [3,4,7,9]. The mechanism of oxidation of reduced inorganic sulfur compounds to sulfate, designated sulfur oxidation, was examined in the Gram-negative, neutrophilic, facultatively lithotrophic bacterium *Paracoccus pantotrophus* GB17. This strain is able to grow with thiosulfate or molecular hydrogen as the energy source and with a large variety of carbon sources [13,18,19].

Analysis of the gene region encoding sulfur-oxidizing ability (Sox) of *P. pantotrophus* and analysis of the proteins involved allowed the assignment of the respective genes to protein functions in this strain and in other Sox bacteria [7]. The enzyme system of *P. pantotrophus* is located in the periplasm, consists of four proteins, SoxXA, SoxYZ, SoxB, and SoxCD, and accepts hydrogen sulfide, sulfur, thiosulfate and sulfite for reduction of horse heart cytochrome *c*. No free intermediate can be detected suggesting that sulfur oxidation is enzyme-bound [7,20]. SoxXA is a heterodimeric *c*-type cytochrome with SoxX (14 216 Da) as monoheme and SoxA (29 352 Da) as diheme subunit. Within the reconstituted enzyme system SoxXA may act as a heme enzyme ligating in an oxidative reaction SoxY with the sulfur substrate or with SoxZ, and act as specific electron mediator. The monomeric dimanganese SoxB (58 611 Da) exhibits a significant similarity to 5′-nucleotidases, but is proposed to function as sulfate thiol esterase [7]. The $\alpha_2\beta_2$-heterotetramer SoxCD is composed of the molybdoprotein SoxC (43 442 Da) and the diheme *c*-type cytochrome SoxD (37 637 Da) [17]. In the reconstituted enzyme system, SoxCD increases the electron yield of thiosulfate oxidation from 2 to 8 mol electrons per mol of thiosulfate [6,17], and thus functions as sulfur dehydrogenase. The heterodimeric SoxYZ is composed of SoxY (10 977 Da) and SoxZ (11 719 Da). SoxYZ does not contain a co-factor or metal. For SoxY, a signal peptide with a twin arginine motif is predicted, while SoxZ has no signal peptide. SoxY and SoxZ of *P. pantotrophus* each contain a single cysteine. A function of the cysteine residues for co-transport of both proteins to the periplasm via covalent linkage appears not likely since SoxZ homologs of other sources do not contain a cysteine residue.

The Sox system of *Paracoccus versutus* (formerly *Thiobacillus versutus* [8]) is closely related to that of *P. pantotrophus* and the lack of free intermediates of the Sox systems of both strains suggested a protein-bound sulfur oxidation at one of the different proteins involved [5,9]. The single cysteine residues of SoxB, SoxY and SoxZ of *P. pantotrophus* were considered as candidates for protein-bound sulfur oxidation [5,6,9]. However, from genomic data of Sox bacteria, a novel motif was identified at the carboxy-terminus of SoxY homologous proteins, (V/I)KV(T/S)(V/I)GGC, and this cysteine residue was suggested as the site of protein-bound sulfur oxidation in aerobic and anaerobic Sox bacteria [7].

We here report evidence that the cysteine residue of SoxY of *P. pantotrophus* functions as the site of oxidation of sulfur to sulfate. Since SoxY proteins are generally present in Sox bacteria, this finding may indicate a common mechanism of sulfur oxidation.

2. Materials and methods

2.1. Purification of the Sox proteins of P. pantotrophus

P. pantotrophus was cultivated lithoautotrophically with thiosulfate at 30°C, concentrated by cross-flow filtration, harvested by centrifugation and stored at −20°C. Cells were resuspended in 25 mM sodium–potassium phosphate buffer, pH 6.5, containing stabilizing ad-

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Abbreviations: NEM, *N*-ethylmaleimide; TCEP, tris(2-carboxyethyl)-phosphine

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ditives (2 mM sodium thiosulfate, 1 mM magnesium sulfate and 1 μM phenylmethylsulfonyl fluoride), and disrupted with a French pressure cell. The resulting extract was subjected to differential centrifugation [17]. SoxYZ was purified from the soluble fraction after ammonium sulfate precipitation and column chromatography. SoxYZ was eluted from the Q Sepharose column with stabilizing buffer specified above. SoxYZ was homogeneous after concentration of the Q Sepharose eluate by Amicon filtration and chromatography on phenyl Sepharose as judged by denaturing sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), as detailed previously [6].

The *c*-type cytochrome SoxXA, the dimanganese SoxB protein and the molybdoprotein SoxCD were purified to homogeneity as described [6,17].

When required, additions like thiosulfate were removed from the protein preparations by repeated rapid dialysis with centricon tubes (Millipore, Bedford, MA, USA) using the buffer without the respective addition.

2.2. Enzyme assays

The assay (1.0 ml) for thiosulfate-dependent cytochrome *c* reduction contained 50 μmol of sodium–potassium phosphate buffer, pH 7.5, 35 nmol of horse heart cytochrome *c*, 0.5 nmol of each SoxXA, SoxYZ, SoxB, and 0.25 nmol SoxCD. Assays for hydrogen sulfide-dependent cytochrome *c* reduction contained 50 mM sodium–potassium phosphate buffer, pH 6.0. A crystal of sodium sulfide washed with oxygen-free water was dissolved, the solution was adjusted to 1 mM and kept under argon. The reaction was started by adding either 100 nmol of sodium thiosulfate, 100 nmol disodium sulfite, or 10 nmol disodium sulfide. The concentrations of the homogeneous Sox-proteins were 4.0 mg SoxXA/ml, 0.89 mg SoxYZ/ml, 3.6 mg SoxB/ml, and 29.0 mg SoxCD/ml. When indicated Sox-proteins were pretreated with an aliquot of 10 mM *N*-ethylmaleimide (NEM) to give a final concentration of 1 mM NEM and the volume added to the assays was compensated for the 1:1.1 dilution.

Protein was determined according to Bradford [2].

2.3. Analytical procedures

The molecular masses of native proteins were determined by non-denaturing PAGE using a linear gradient of 5–27.5% polyacrylamide [1]. The molecular masses of denatured proteins were determined by SDS–PAGE according to Laemmli [10]. Proteins were stained with Coomassie blue [22].

Molecular masses of proteins were determined by nano-electrospray mass spectrometry with a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanoflow-Z-spray ion source [15]. The masses of proteins were also determined by matrix-assisted laser desorption ionization (MALDI; Voyager, Perseptive Biosystems, Manchester, UK) at the facilities of the Max-Planck-Institut, Dortmund. Peptides of tryptic digested SoxYZ were determined at the facilities of the Max-Delbrück-Centrum, Berlin. The probability for signal-peptide cleavage sites were determined according to Nielsen et al. [14].

3. Results

3.1. Electrospray ionization mass spectrometry of SoxYZ

The molecular masses for the mature SoxY and SoxZ as deduced from *soxY* and *soxZ* were 10 977 and 11 719 Da, respectively [6]. Electrospray ionization mass spectrometry of a homogeneous SoxYZ preparation, as judged from SDS-PAGE, yielded masses of 11 094, 11 119, 11 717, and 11 816 Da (Fig. 1). The mass of 11 717 Da was assigned to SoxZ and was identical to the mass deduced from the amino acid sequence. The difference of 117 and 142 Da for SoxY might have indicated a covalent addition of $[S_2O_3]$ (112 Da) and of $[S_3O_3]$ (144 Da). A minor signal of 11 816 Da matched precisely with SoxY matured by the signal peptidase at a cleavage site predicted to occur after amino acid 19 of SoxY, albeit with a low probability. SoxY is mainly cleaved after amino acid 28, as confirmed by determination of the amino acid sequence of the N-terminus [6]. Also, a minor

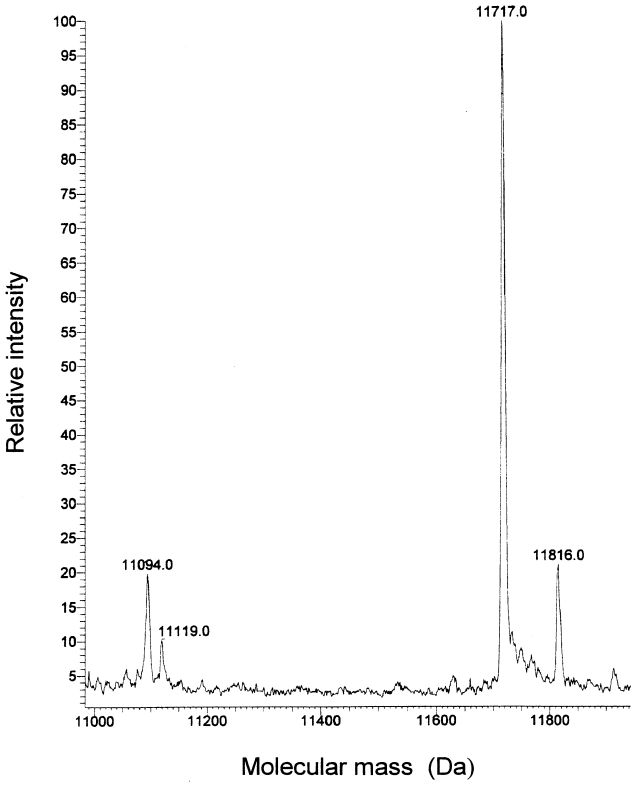

Fig. 1. Electrospray ionization mass spectrometry of SoxYZ. SoxYZ was desalted by gel filtration and concentrated by ultrafiltration with centricon tubes to 3.0 mg/ml. SoxYZ (1 μl) was mixed with 9 μl of a mixture of 5% (v/v) of formic acid and 50% (v/v) of methanol. A sample (4 μl) was analyzed as detailed by Prinz et al. [15].

signal of 0.2% of intensity was observed for 22 693 Da, and of 1.4% of intensity for 23 437 Da (data not shown). These masses matched those calculated for covalently bound SoxYZ and SoxZZ. SoxY and SoxZ each contain a single cysteine. The masses suggested a covalent linkage of the subunits probably via a protein disulfide bond, and these bonds are not disrupted by the method applied. Such a disulfide bond may be integral to the aerobic lithotrophic sulfur oxidation of *P. pantotrophus* or an artefact during the aerobic purification procedure.

3.2. Multiple masses of SoxY

Electrospray mass spectrometry yielded weak signals for SoxY of different molecular masses. These results prompted us to examine SoxYZ by MALDI mass spectrometry. From the 'as isolated' SoxYZ preparation, which contains 2 mM thiosulfate, one mass of 11 719 Da was determined representing SoxZ (Fig. 2a). However, different masses were obtained for SoxY with minor peaks at 10 977, 11 009, 11 089 and 11 122 Da, and a major peak at 11 056 Da (Fig. 2a). The observed differences of +32, +79, +112 and +145 Da suggested the addition of sulfur compounds to SoxY, and [S], $[SO_3]$, $[S_2O_3]$, and $[S_3O_3]$ would account for the mass of SoxY +32, +80, +112 and +144 Da, respectively. To examine the chemical nature of the bond of the adducts to SoxY, SoxYZ was treated with substrate and non-substrate reductants.

Disulfide bonds are cleaved by reducing agents at room temperature. Prior to reduction, thiosulfate was removed

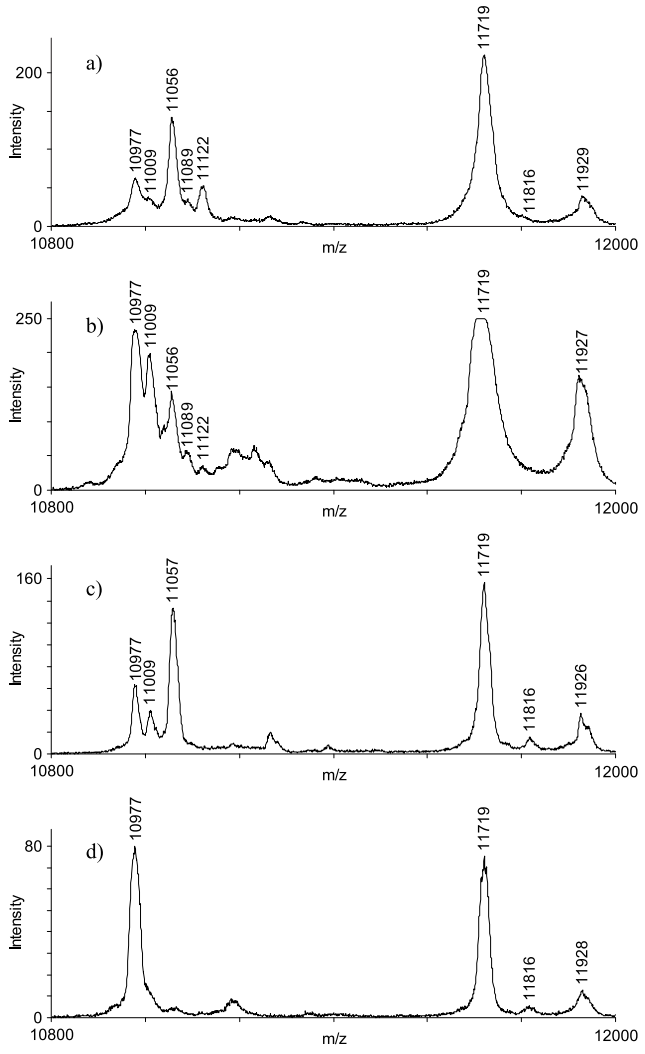

Fig. 2. MALDI mass spectrometry of SoxYZ. 1 μl of SoxYZ (0.36 mg/ml) was added to 9 μl of a solution containing saturated sinapinic acid in 50% (v/v) trifluoro acetic acid and 50% (v/v) acetonitrile, and subjected to a MALDI-slide and dried prior to analysis. a: SoxYZ 'as isolated'; b: SoxYZ with 1 mM sodium sulfide; c: SoxYZ with 1 mM sodium sulfite; d: SoxYZ with 1 mM tris(carboxyethyl) phosphine.

from the 'as isolated' SoxYZ preparation by rapid dialysis, and SoxYZ was then reduced by the addition of 1 mM sodium sulfide. MALDI mass spectrometry of this preparation revealed again a single peak representing the mass of SoxZ (Fig. 2b). From SoxY, the main peak was at 10 977 Da, with other species at 11 009, 11 056, 11 089 and 11 122 Da in decreasing signal intensities (Fig. 2b). Addition of sodium sulfite (1 mM) to SoxYZ resulted in a major mass of 11 057 Da, indicating a [SO_3] adduct (Fig. 2c). Reduction by tris(2-carboxyethyl)phosphine (TCEP) resulted in one major species of 10 977 Da and only traces of other masses (Fig. 2d). The mass of SoxZ was not affected by the treatment with sulfur substrates or non-sulfur reductants. The mass of 11 816 (Fig. 2a–d) was assigned to SoxY processed after the alanine-19 residue, and the mass of 11 928 ± 2 Da (Fig. 2a–d) matched that species plus the mass of thiosulfate (112 Da).

Tryptic digest of SoxY yields the octapeptide VTIGGCGG [6]. However, MALDI mass spectrometry of the peptides resulting from tryptic digest did not yield a mass to be assigned to the octapeptide or masses of its derivative (data not shown).

3.3. Significance of free sulfhydryl moieties of the Sox proteins

To examine if free sulfhydryl moieties of cysteine residues of the Sox proteins were essential for the catalytic activity, SoxXA, SoxYZ, SoxB, and SoxCD were separately incubated with 1 mM NEM prior to their addition to the assay and reconstitution of the Sox enzyme system. NEM covalently binds to sulfhydryl moieties of proteins. Thiosulfate-dependent cytochrome *c* reduction by the reconstituted Sox enzyme system of *P. pantotrophus* proceeded at a linear rate (Fig. 3). A linear reaction rate was also observed when each of the Sox proteins was pretreated with NEM, albeit at rates decreased by 6.5–33.4% (Table 1). Again, when the four NEM-pretreated proteins were added to the assay, the reaction was linear with time and the reaction rate was decreased by 60.4%. The partial inactivation of the Sox proteins appeared as cumulative inhibition, and the residual catalytic activity of the NEM-pretreated proteins of 39.6% was close to the activity calculated from the individual degrees of inactivation (37.9%).

Upon introduction of the four NEM-pretreated proteins to the assay, NEM was introduced at a concentration of 20 μM. At this concentration, the thiosulfate-dependent cytochrome *c* reduction rate was inhibited by 12.5% (Table 1). When the Sox proteins were not pretreated, but NEM (1 mM) was added to the assay, the initial rate was inhibited by 85.4% (Table 1) ceasing gradually with time, as shown for the time course of inhibition with 0.50 mM NEM (Fig. 3).

SoxYZ was reduced by pretreatment with 1 mM TCEP, which resulted in an almost complete inhibition of the reconstituted thiosulfate-dependent cytochrome *c* reduction rate

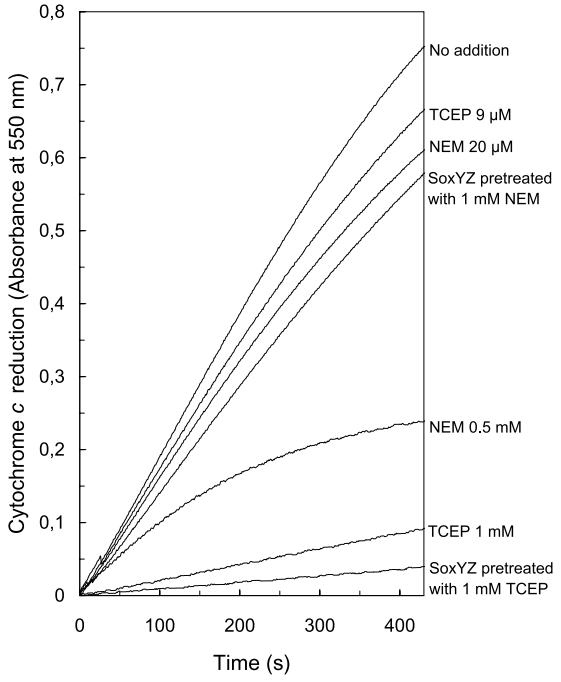

Fig. 3. Thiosulfate-dependent cytochrome *c* reduction by the reconstituted Sox enzyme system of *P. pantotrophus* in the presence of different inhibitors. SoxYZ was pretreated with 1 mM NEM or TCEP for 30 min at 30°C prior to addition to the assay. The principal conditions and concentrations of the inhibitors are indicated.

Table 1
Reaction rates of the reconstituted thiosulfate-oxidizing enzyme system of *P. pantotrophus* with NEM-treated Sox proteins

Sox protein pretreated with NEM	NEM concentration in the assay (μM)	Thiosulfate-dependent cytochrome *c* reduction (nmol/min)	Relative activity (%)
None	0	4.17	100
None	20	3.65	87.5
None	1000	0.61	14.6
XA, YZ, B, CD	20	1.65	39.6
XA	4	3.90	93.5
YZ	9	3.16	75.8
B	6	3.34	80.1
CD	1	2.78	66.7

(Fig. 3). Also, addition of 1 mM TCEP to the assay inhibited the thiosulfate oxidation almost completely (Fig. 3). These data indicated that SoxYZ changed the oxidation state in the course of the reaction. At 1 mM TCEP, the final electron acceptor horse heart cytochrome *c* was not reduced spontaneously.

4. Discussion

We have presented evidence that the SoxY subunit of the SoxYZ protein of the Sox enzyme system of *P. pantotrophus* is the site of lithotrophic sulfur oxidation. First, electrospray ionization spectrometry revealed masses equivalent to those of the adducts of thiosulfate [S_2O_3] and thioperoxomonosulfate [S_3O_3] to SoxY. Moreover, multiple masses of the SoxY protein 'as isolated' were observed, as determined by MALDI-TOF spectrometry, indicating free SoxY and additional masses suggest adducts of [S], [SO_3], [S_2O_3], and [S_3O_3]. Second, these adducts were removed from SoxYZ upon reduction of the protein, indicating a linkage of the additional masses via a thioether or thioester bond. Third, agents blocking free sulfhydryl moieties and those keeping sulfhydryl moieties in the reduced state inhibited the reconstituted Sox system, indicating the significance of sulfhydryls within the reaction cycle.

The Sox multienzyme system of *P. versutus* [11] is considered very similar to that of *P. pantotrophus* [5,7], and enzyme A is equivalent to SoxZ [6]. From equilibrium dialysis, enzyme A was shown to bind thiosulfate ($K_d = 70$ μM) with sulfite as competitive inhibitor ($K_i = 25$ μM) [12], indicating that binding of thiosulfate to the enzyme could be through the sulfonate ($-SO_3^-$) group rather than the sulphane ($-S^-$) group of the thiosulfate [12]. On the basis of this observation and the relatedness of the systems, it is suggested that the sulfur substrates may be coordinated by SoxZ and covalently bound to SoxY of *P. pantotrophus*.

The methods applied here demonstrated that the additional masses of SoxY of +32, +79, +112 and +145 were linked to the polypeptide either via a thioester or thioether bond. The Sox enzyme system of *P. pantotrophus* yields 8 mol of electrons per mol of thiosulfate. These are transferred to horse heart cytochrome *c* according to Eq. 1 [6].

$$S_2O_3^{2-} + 5\ H_2O + 8\ \text{cyt}\ c_{ox} \rightarrow 2\ SO_4^{2-} + 10\ H^+ + 8\ \text{cyt}\ c_{red} \quad (1)$$

Since oxidation of thiosulfate to sulfate yields eight electrons, two electrons may be released from thiosulfate upon an oxidation reaction to link thiosulfate to the sulfhydryl moiety of the cysteine residue or from protein disulfide formation involving SoxY. The sulfane–sulfur may then be added to the disulfide bond to yield *S*-thiocysteinesulfate. Formation of *S*-thiocysteine may have resulted from the subsequent hydrolysis to release sulfate as given in Eqs. 2 and 3, and the respective masses of SoxY have been identified by MALDI mass spectrometry. The equilibrium of such initial reaction would be shifted to the substrate side upon reduction, and lead to the inhibition observed with TCEP.

$$\text{SoxY}-S^- + S_2O_3^{2-} \rightarrow \text{SoxY}-S-S-SO_3^- + 2H^+ + 2e^- \quad (2)$$

$$\text{SoxY}-S-S-SO_3^- + H_2O \rightarrow \text{SoxY}-S-S^- + H_2SO_4^{2-} \quad (3)$$

$$\text{SoxY}-S-S^- + 3\ H_2O \rightarrow \text{SoxY}-S-SO_3^- + 6H^+ + 6e^- \quad (4)$$

The oxidation state of the outer sulfur atom of *S*-thiocysteine is zero and that of *S*-cysteinesulfate is +6. Thus, the oxidation of *S*-thiocysteine to *S*-thiocysteinesulfate yields six electrons and SoxCD is proposed to act on the outer sulfur atom of *S*-thiocysteine (Eq. 4), thus functioning as sulfur dehydrogenase [7].

The masses being in accordance with *S*-thiocysteine and *S*-thiocysteinesulfate of SoxY were identified by MALDI mass spectrometry. The observed mass of SoxY +144 Da may have resulted from the addition of thiosulfate to *S*-thiocysteine (Eq. 5). This adduct would be identical with that of thioperoxomonosulfate ($[O_3S-S-S]^{2-}$) to free SoxY (Eq. 6). In polythionate-utilizing bacteria, tetrathionate is probably hydrolyzed by tetrathionate hydrolase to sulfate and thioperoxomonosulfate [16]. The latter compound or the products of its spontaneous decomposition (e.g. sulfur and thiosulfate [21]) may be oxidized by a similar system, as described for *P. pantotrophus*.

$$\text{SoxY}-S-S^- + S_2O_3^{2-} \rightarrow \text{SoxY}-S-S-S-SO_3^- + 2e^- \quad (5)$$

$$\text{SoxY}-S^- + S_3O_3^{2-} \rightarrow \text{SoxY}-S-S-S-SO_3^- + 2e^- \quad (6)$$

The Sox enzyme system of *P. pantotrophus* is most active with sodium sulfide and least active with sodium sulfite [20]. NEM binds covalently to free sulfhydryl groups. When each of the resting proteins of the enzyme system was pretreated with NEM, the catalytic activity was partially inactivated rather than inhibited, depending on the individual sensitivity of the different Sox proteins. Addition of NEM to the assay caused complete cessation of the activity, depending on the concentration of NEM and duration of the reaction. These kinetics clearly indicated a turn-over-dependent inactivation, and thus, the occurrence of a crucial free thiol during the reaction cycle.

When the enzyme system was reduced with a non-substrate reductant such as TCEP, the thiosulfate oxidation was almost completely inhibited, and this observation indicated the requirement of an oxidation reaction of a thiol.

The sulfur binding motif at the carboxy-terminal end of SoxY is highly conserved in Sox bacteria. Therefore, the principal mechanism of bacterial oxidation of inorganic sulfur to sulfate may proceed similarly in aerobic lithotrophic and anaerobic phototrophic bacteria.

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References

[1] Andersson, L.O., Borg, H. and Mikaelsson, M. (1972) FEBS Lett. 20, 199–202.
[2] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[3] Brune, D.C. (1995) in: Anoxygenic photosynthetic bacteria (Blankenship, R.E., Madigan, M.T. and Bauer, C.E., Eds.), pp. 847–870. Kluwer, Doordrecht.
[4] deZwart, J.M.M., Nelisse, P.N. and Kuenen, J.G. (1996) FEMS Microbiol. Ecol. 20, 261–270.
[5] Friedrich, C.G. (1998) Adv. Microb. Physiol. 39, 235–289.
[6] Friedrich, C.G., Quentmeier, A., Bardischewsky, F., Rother, D., Kraft, R., Kostka, S. and Prinz, H. (2000) J. Bacteriol. 182, 4677–4687.
[7] Friedrich, C.G., Rother, D., Bardischewsky, F., Quentmeier, A. and Fischer, J. (2001) Appl. Environ. Microbiol. 67, 2873–2882.
[8] Katayama, Y., Hiraishi, A. and Kuraishi, H. (1995) Microbiology 141, 1469–1477.
[9] Kelly, D.P., Shergill, J.K., Lu, W.-P. and Wood, A.P. (1997) Antonie van Leeuwenhoek 71, 95–107.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Lu, W.-P. and Kelly, D.P. (1983) J. Gen. Microbiol. 129, 3549–3564.
[12] Lu, W.-P., Swoboda, B.E.P. and Kelly, D.P. (1985) Biochim. Biophys. Acta 828, 116–122.
[13] Ludwig, W., Mittenhuber, G. and Friedrich, C.G. (1993) Int. J. Syst. Bacteriol. 43, 363–367.
[14] Nielsen, H., Engelbrecht, J., Brunack, S. and von Heijne, G. (1997) Protein Eng. 10, 1–6.
[15] Prinz, H., Lavie, A., Scheidig, A., Spangenberg, O. and Konrad, M. (1999) J. Biol. Chem. 274, 35337–35342.
[16] Pronk, J.T., Meulenberg, R., Hazeu, W., Bos, P. and Kuenen, J.G. (1990) FEMS Microbiol. Rev. 75, 293–306.
[17] Quentmeier, A., Kraft, R., Kostka, S., Klockenkämper, R. and Friedrich, C.G. (2000) Arch. Microbiol. 173, 117–125.
[18] Rainey, F.A., Kelly, D.P., Stackebrandt, E., Burghardt, J., Hiraishi, A., Katayama, Y. and Wood, A.P. (1999) Int. J. Syst. Bacteriol. 49, 645–651.
[19] Robertson, L.A. and Kuenen, J.G. (1983) J. Gen. Microbiol. 129, 2847–2855.
[20] Rother, D., Henrich, H.-J., Quentmeier, A., Bardischewsky, F. and Friedrich, C.G. (2001) J. Bacteriol., in press.
[21] Steudel, R., Holdt, G., Göbel, T. and Hazeu, W. (1987) Ang. Chem. 26, 151–153.
[22] Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods Enzymol. 26, 3–27.